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**FIRST CLASS MAIL CERTIFICATE**

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*Linda O. Palladino*  
Linda O. Palladino

July 24, 2001  
Date

Att. Docket No. REG 710B



**THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application Of : Xia, et al.  
USSN : 09/773,877  
Filed : January 31, 2001  
For : METHODS OF TREATING  
INFLAMMATORY SKIN DISEASES

July 24, 2001

Commissioner of Patents  
U.S. Patent and Trademark Office  
Washington, D.C. 20231

**PRELIMINARY AMENDMENT, RESPONSE TO  
NOTICE TO FILE MISSING PARTS, AND PETITION FOR ONE MONTH  
EXTENSION OF TIME**

Sir:

On May 10, 2001, the U.S. Patent and Trademark Office issued a Notice to File Missing Parts of Nonprovisional Application ("Notice"). A response to the Notice was due on July 10, 2001. Applicants hereby request a one month extension of time and a check in the amount of \$110.00 is enclosed herewith for the required fee. A response to the May 10, 2001 Notice is therefore due on August 10, 2001, and this response is being timely filed.

In response to the May 10, 2001 Notice, applicants enclose the following: Exhibit A: a copy of the Notice to File Missing Parts dated May 10, 2001; Exhibit B: executed Declaration and Power of Attorney; Exhibit C: Formal Drawings (58 sheets); Exhibit D: Sequence Listing in paper and computer readable forms pursuant to 37 C.F.R

08/02/2001 AZERBANI 00000085 09773877

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§1.821(c) and (e) respectively; Exhibit E: Marked-up Version of amended pages 11, 13, 15, 24, 25, 26, 27, 30, 57-60, 66-70, 74, and 88; Exhibit F: Substitute Sheets for amended pages 11, 13, 15, 24, 25, 26, 27, 30, 57-60, 66-70, 74, and 88; A check in the amount of \$130.00 for the required surcharge of \$130.00 is enclosed herewith.

Prior to examination of the subject application on the merits, please amend the application as follows:

**IN THE SPECIFICATION:**

On page 11, line 20, after "Figure 13A-13D" insert --(SEQ ID NOS: 13 and 14)--.  
On page 11, line 21, after "Figure 14A-14C" insert --(SEQ ID NOS: 15 and 16)--.  
On page 11, line 22, after "Figure 15A-15C" insert --(SEQ ID NOS: 17 and 18)--.  
On page 11, line 23, after "Figure 16A-16D" insert --(SEQ ID NOS: 19 and 20)--.  
On page 11, line 24, after "Figure 21A-21C" insert --(SEQ ID NOS: 21 and 22)--.  
On page 11, line 25, after "Figure 22A-22C" insert --(SEQ ID NOS: 23 and 24)--.  
On page 11, line 26, after "Figure 24A-24C" insert --(SEQ ID NOS: 25 and 26)--.  
On page 13, line 6, after "Figure 10A-10D" insert --(SEQ ID NOS: 11 and 12)--.  
On page 13, line 6, after "Figure 24A-24C" insert --(SEQ ID NOS: 25 and 26)--.  
On page 15, line 19, after "Figure 13A-13D" insert --(SEQ ID NOS: 13 and 14)--.  
On page 15, line 20, after "Figure 14A-14C" insert --(SEQ ID NOS: 15 and 16)--.  
On page 15, line 21, after "15A-15C" insert --(SEQ ID NOS: 17 and 18)--.  
On page 15, line 21, after "Figure 16A-16D" insert --(SEQ ID NOS: 19 and 20)--.  
On page 15, line 22, after "Figure 21A-21C" insert --(SEQ ID NOS: 21 and 22)--.  
On page 15, line 23, after "Figure 22A-22C" insert --(SEQ ID NOS: 23 and 24)--.  
On page 15, line 24, after "Figure 24A-24C" insert --(SEQ ID NOS: 25 and 26)--.  
On page 24, line 18, after "Figure 10A-10D" insert --(SEQ ID NOS: 11 and 12)--.  
On page 24, line 26, after "Figure 13A-13D" insert --(SEQ ID NOS: 13 and 14)--.  
On page 24, line 28, after "Figure 14A-14C" insert --(SEQ ID NOS: 15 and 16)--.  
On page 25, line 3, after "Figure 15A-15C" insert --(SEQ ID NOS: 17 and 18)--.

On page 25, line 6, after "**Figure 16A-16D**" insert --(SEQ ID NOS: 19 and 20)--.

On page 26, line 22, after "**Figure 21A-21C**" insert --(SEQ ID NOS: 21 and 22)--.

On page 27, line 1, after "**Figure 22A-22C**" insert --(SEQ ID NOS: 23 and 24)--.

On page 27, line 9, after "**Figure 24A-24C**" insert --(SEQ ID NOS: 25 and 26)--.

On page 30, line 20, after "**Figure 36**" insert --(SEQ ID NO: 27)--.

On page 57, line 14, after "Figure 10A-10D" insert --(SEQ ID NOS: 11 and 12)--.

On page 58, line 4, after "Figure 10A-10D" insert --(SEQ ID NOS: 11 and 12)--.

On page 59, line 1, after "Figure 10A-10D" insert --(SEQ ID NOS: 11 and 12)--.

On page 59, line 8, after "Figure 13A-13D" insert --(SEQ ID NOS: 13 AND 14)--.

On page 59, line 15, after "Figure 10A-10D" insert --(SEQ ID NOS: 11 and 12)--.

On page 60, line 1, after "Figure 14A-14C" insert --(SEQ ID NOS: 15 and 16)--.

On page 60, line 12, after "15A-15C" insert --(SEQ ID NOS: 17 and 18)--.

On page 60, line 21, after "Figure 10A-10D" insert --(SEQ ID NOS: 11 and 12)--.

On page 60, line 25, after "Figure 16A-16D" insert --(SEQ ID NOS: 19 and 20)--.

On page 66, line 17, after "3'" insert --(SEQ ID NO: 1)--.

On page 66, line 19, after "3'" insert --(SEQ ID NO: 2)--.

On page 66, line 22, after "Figure 21A-21C" insert --(SEQ ID NOS: 21 and 22)--.

On page 67, line 1, after "Figure 21A-21C" insert --(SEQ ID NOS: 21 and 22)--.

On page 67, line 2, after "Figure 21A-21C" insert --(SEQ ID NOS: 21 and 22)--.

On page 67, line 8, after "G-3'" insert --SEQ ID NO: 3)--.

On page 67, line 11, after "AAATG-3'" insert --(SEQ ID NO: 4)--.

On page 67, line 17, after "21A-21C)" insert --(SEQ ID NOS: 21 and 22)--.

On page 67, line 20, after "Figure 21A-21C" insert --(SEQ ID NOS: 21 and 22)--.

On page 68, line 11, after "Figure 21A-21C" insert --(SEQ ID NOS: 21 and 22)--.

On page 68, line 24, after "-3'" insert --(SEQ ID NO: 5)--.

On page 69, line 2, after "TTGGT)" insert --(SEQ ID NO: 6)--.

On page 69, line 6, after "Figure 22A-22C" insert --(SEQ ID NOS: 23 and 24)--.

On page 69, line 10, after "Figure 22A-22C" insert --(SEQ ID NOS: 23 and 24)--.

On page 69, line 11, after "Figure 22A-22C" insert --(SEQ ID NOS: 23 and 24)--.

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On page 69, line 16, after

“(ATCCAGCTGTTGCCCAGGAAGTCGCTGGAGCTGCTGGTA)” insert --(SEQ ID NO: 7)--.

On page 69, line 17, after

“(ATTTTCATGCACAATGACCTCGGTGCTCTCCCGAAATCG)” insert --(SEQ ID NO: 8)--.

On page 70, line 6, after

“(TCATAGATATCCAGCTGTTGCCCAGGAGTCGCTGGAG)” insert --(SEQ ID NO: 9)--.

On page 70, line 9, after

“(GATAATGCCCCGGGCCATTTTCATGCACAATGACCTCGGT)” insert --(SEQ ID NO: 10)--.

On page 70, line 15, after “Figure 22A-22C” insert --(SEQ ID NOS: 23 and 24)--.

On page 70, line 18, after “Figure 22A-22C” insert --(SEQ ID NOS: 23 and 24)--.

On page 74, line 1, after “Figure 24A-24C” insert --(SEQ ID NOS: 25 and 26)--.

On page 88, line 2, after “Figure 36” insert --(SEQ ID NO: 27)--.

On page 88, line 20, delete “isobserved” and insert --is observed--.

On page 88, line 22, after “Figure 36” insert --(SEQ ID NO: 27)--.

Applicants direct the subject Sequence Listings submitted herewith be added to the specification.

I hereby state that the content of the paper and computer readable copies of the Sequence Listing, submitted in accordance with 37 C.F.R §1.821(c) and (e) respectively, are the same. I hereby state that the content of the paper and computer readable copies of the Sequence Listing submitted herewith and referred to herein in accordance with 37 C.F.R. § 1.821(g), contain no new matter.

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No additional fee is deemed necessary. However, if any additional fee is required, the Commissioner is hereby authorized to charge any such fee to Deposit Account No. 18-0650.

Respectfully submitted,



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upstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

In still another preferred embodiment, the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

In a preferred embodiment of the invention, the multimerizing component comprises an immunoglobulin domain.

In another embodiment, the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Preferred embodiments include an isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic acid molecule consists of a nucleotide sequence selected from the group consisting of

- (a) the nucleotide sequence set forth in Figure 13A-13D, (SEQ ID NOS: 13 AND 14)
- (b) the nucleotide sequence set forth in Figure 14A-14C, (SEQ ID NOS: 15 AND 16)
- (c) the nucleotide sequence set forth in Figure 15A-15C, (SEQ ID NOS: 17 AND 18)
- (d) the nucleotide sequence set forth in Figure 16A-16D, (SEQ ID NOS: 19 AND 20)
- (e) the nucleotide sequence set forth in Figure 21A-21C, (SEQ ID NOS: 21 AND 22)
- (f) the nucleotide sequence set forth in Figure 22A-22C, (SEQ ID NOS: 23 AND 24)
- (g) the nucleotide sequence set forth in Figure 24A-24C; and (SEQ ID NOS: 25 AND 26)
- (h) a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a), (b), (c), (d),

polypeptide which comprises growing cells of the host-vector system under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

- 5 Additional embodiments include a fusion polypeptide encoded by the nucleic acid sequence set forth in Figure 10A-10D<sup>(SEQ ID NOS: 11 AND 12)</sup> or Figure 24A-24C<sup>(SEQ ID NOS: 25 AND 26)</sup> which has been modified by acetylation or pegylation wherein the acetylation is accomplished with at least about a 100 fold molar excess of acetylation reagent or wherein acetylation is accomplished with a molar
- 10 excess of acetylation reagent ranging from at least about a 10 fold molar excess to about a 100 fold molar excess or wherein the pegylation is 10K or 20K PEG.

- 15 A preferred embodiment includes a method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal the fusion polypeptide described above, including embodiments wherein the mammal is a human, the fusion polypeptide is acetylated or the fusion polypeptide is pegylated.

- 20 A further embodiments is a fusion polypeptide which specifically binds the VEGF receptor ligand VEGF.

- 25 A preferred embodiment of the invention is a method of blocking blood vessel growth in a human comprising administering an effective amount of the fusion polypeptide described above.

Also preferred is a method of inhibiting VEGF receptor ligand activity in a mammal comprising administering to the mammal an effective amount of

## MARKED-UP VERSION

Preferred embodiments include a fusion polypeptide wherein amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor and a fusion polypeptide wherein the amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor.

In yet another embodiment, the fusion polypeptide multimerizing component comprises an immunoglobulin domain including an embodiment wherein the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Preferred embodiments include a fusion polypeptide comprising an amino acid sequence of a modified Flt1 receptor, wherein the amino acid sequence selected from the group consisting of (a) the amino acid sequence set forth in Figure 13A-13D<sup>(SEQ ID NOS: 13 AND 14)</sup>; (b) the amino acid sequence set forth in Figure 14A-14C<sup>(SEQ ID NOS: 15 AND 16)</sup>; (c) the amino acid sequence set forth in Figure 15A-15C<sup>(SEQ ID NOS: 17 AND 18)</sup>; (d) the amino acid sequence set forth in Figure 16A-16D<sup>(SEQ ID NOS: 19 AND 20)</sup>; (e) the amino acid sequence set forth in Figure 21A-21C<sup>(SEQ ID NOS: 21 AND 22)</sup>; (f) the amino acid sequence set forth in Figure 22A-22C<sup>(SEQ ID NOS: 23 AND 24)</sup>; and (g) the amino acid sequence set forth in Figure 24A-24C<sup>(SEQ ID NOS: 25 AND 26)</sup>.

Another preferred embodiment is a method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal the fusion polypeptide described above.



greater than a 1:1 stoichiometric ratio, both the Flt1(1-3)-Fc and the step-acetylated Flt1(1-3)-Fc proteins are able to saturate the VEGF, regardless of the degree of acetylation.

- 5 **Figure 9.** Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc. Balb/c mice (23-28g) were injected subcutaneously with 4mg/kg of unmodified or 10, 20, 40, 60 and 100 fold excess samples of step-acetylated Flt1(1-3)-Fc (3 mice for unmodified, 10, 20 and 40 fold excess samples and 2 mice for 60 and 100 fold excess samples). The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days and 3 days after injection. The sera were assayed in an ELISA-based assay designed to detect Flt1(1-3)-Fc. The  $T_{max}$  for all of the Flt1(1-3)-Fc proteins tested was at the 6 hour time point but the  $C_{max}$  was as follows: Unmodified Flt1(1-3)-Fc: 0.06µg/ml; 10 fold excess sample: - 0.7µg/ml, 15 20 fold excess sample - 2µg/ml, 40 fold excess sample - 4µg/ml, 60 fold excess sample - 2µg/ml, 100 fold excess sample - 1µg/ml.

(SEQ ID NOS: 11 AND 12)

**Figure 10A-10D.** Nucleic acid and deduced amino acid sequence of Flt1(1-3)-Fc.

**Figure 11.** Schematic diagram of the structure of Flt1.

**Figure 12A and 12B.** Hydrophilicity analysis of the amino acid sequences of Ig domain 2 and Ig domain 3 of Flt1.

(SEQ ID NOS: 13 AND 14)

**Figure 13A-13D.** Nucleic acid and deduced amino acid sequence of Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc.

(SEQ ID NOS: 15 AND 16)

**Figure 14A-14 C.** Nucleic acid and deduced amino acid sequence of

Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc.

(SEQ ID NOS: 17 AND 18)  
**Figure 15A-15C.** Nucleic acid and deduced amino acid sequence of  
 Mut3: Flt1(2-3)-Fc.

5 (SEQ ID NOS: 19 AND 20)  
**Figure 16A-16D.** Nucleic acid and deduced amino acid sequence of  
 Mut4: Flt1(1-3<sub>R->N</sub>)-Fc.

**Figure 17.** Binding of unmodified Flt1(1-3)-Fc, basic region deletion  
 10 mutant Flt1(1-3)-Fc, and Flt1(1-3)<sub>R->N</sub> mutant proteins in a Biacore-based  
 assay. At the sub-stoichiometric ratio (0.25 μg/ml Flt1(1-3)-Fc of  
 unmodified, acetylated or genetically modified samples vs. 01. μg/ml  
 VEGF), there is insufficient Flt1(1-3)-Fc protein to block binding of VEGF  
 to the Flt1(1-3)-Fc immobilized on the Biacore chip. At 0.5 μg/ml of  
 15 unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, the  
 stoichiometric ratio approximates 1:1 and there is an increased ability to  
 block VEGF binding to the Biacore chip. At 1.0 μg/ml of unmodified,  
 acetylated or genetically modified Flt1(1-3)-Fc proteins, which is  
 approximately a 10:1 stoichiometric ratio, the Flt1(1-3)-Fc proteins are  
 20 able to block binding of VEGF to the Biacore chip, but they are not  
 equivalent. Unmodified, acetylated, and Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc are  
 essentially equal in their ability to block VEGF binding, whereas Mut4:  
 Flt1(1-3<sub>R->N</sub>)-Fc is somewhat less efficient at blocking binding.

25

**Figure 18.** Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc, Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc, and Flt1(2-3) mutant proteins to Matrigel® coated plates. Unmodified Flt1(1-3)-Fc protein binds avidly to these wells, the Mut3: Flt1(2-3)-Fc protein binds somewhat more weakly, the Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc protein binds more weakly still, and the Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc protein shows the best profile, binding more weakly than any of the other mutant proteins. The Mut4: Flt1(1-3<sub>R->N</sub>)-Fc glycosylation mutant protein shows only marginal benefit on the Matrigel assay.

**Figure 19.** Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc, Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc, and Flt1(2-3) mutant proteins in an ELISA-based assay. At the concentrations tested, unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc, Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc, and Flt1(2-3) mutant proteins bind VEGF similarly.

**Figure 20.** Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc, Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc, and Flt1(2-3) mutant proteins. the C<sub>max</sub> for these reagents was as follows: Unmodified Flt1(1-3)-Fc - 0.15μg/ml; 40 fold molar excess acetylated Flt1(1-3)-Fc - 1.5μg/ml; and Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc - 0.7μg/ml.

(SEQ ID NOS: 21 AND 22)

**Figure 21A-21C.** Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed Flt1D2.Flk1D3.FcΔC1(a).

(SEQ ID NOS: 23 AND 24)

**Figure 22A-22C.** Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed Flt1D2.VEGFR3D3.FcΔC1(a).

**Figure 23.** Extracellular Matrix (ECM) Assay. The results of this assay demonstrate that the Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) proteins are considerably less sticky to the ECM as compared to the Flt1(1-3)-Fc protein.

(SEQ ID NOS: 25 AND 26)

**Figure 24A-24C.** Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed VEGFR1R2-FcΔC1(a).

**Figure 25A-25C.** Phosphorylation assay. At a 1.5 molar excess of either Flt1(1-3)-Fc , Flt1(1-3)-Fc (A40) or transient Flt1D2Flk1D3.FcΔC1(a) there is complete blockage of receptor stimulation by these three modified Flt1 receptors as compared to control media challenge. In contrast, transient Flt1D2VEGFR3D3.FcΔC1(a) does not show significant blockage at this molar excess, as compared with VEGF positive control challenge. Similar results are seen in Figure 25B, where the modified Flt receptors are in a 3-fold molar excess to VEGF165 ligand. In Figure 25C, where the modified Flt1 receptors are in a 6-fold molar excess to VEGF165 ligand, transient Flt1D2VEGFR3D3.FcΔC1(a) can now be shown to be partially blocking VEGF165-induced stimulation of cell-surface receptors.

**Figure 26A-26B.** Phosphorylation assay. Detection by Western blot of

loaded onto a Superose 12 PC 3.2/30 equilibrated in 6M GuHCl and eluted. Peak #1 represents Flt1D2Flk1D3.FcΔC1(a) and peak #2 represents VEGF165.

5 **Figure 33, Figure 34 and Figure 35.** Size Exclusion Chromatography (SEC) with On-Line Light Scattering. Size exclusion chromatography column with a MiniDawn on-line light scattering detector (Wyatt Technology, Santa Barbara, California) and refractive index (RI) detectors (Shimadzu, Kyoto, Japan) was used to determine the molecular weight (MW) of the receptor-ligand complex. As shown in Figure 33, the elution profile shows two peaks. Peak #1 represents the receptor-ligand complex and peak #2 represents the unbound VEGF165. MW was calculated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor-ligand complex. The results of these determinations are as follows: MW of the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex at the peak position is 157 300 (Figure 33), the MW of VEGF165 at the peak position is 44 390 (Figure 34) and the MW of R1R2 at the peak is 113 300 (Figure 35).

(SEQ ID NO: 27)

20 **Figure 36.** Peptide mapping and glycosylation analysis. The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) were determined by a peptide mapping method. There are a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to the Fc region. Cys27 is disulfide bonded to Cys76. Cys121 is disulfide bonded to Cys 182. The first two cysteines in the Fc region (Cys211 and Cys214) form

1998, Bioessays 20(2):156-67). In other cases, the known crystal structure of a protein reveals a cluster of positively charged residues on the surface of a protein, but the residues come from different regions of the primary sequence and are only brought together when the protein

5 folds into its tertiary structure. Thus it is difficult to deduce whether an isolated amino acid residue forms part of a cluster of basic residues on the surface of the protein. However, if there is a cluster of positively charged amino acid residues in the primary sequence, it is not unreasonable to surmise that the residues are spatially close to one

10 another and might therefore be part of an extracellular matrix component binding site. Flt1 receptor has been studied extensively and various domains have been described (see for example Tanaka et al., 1997, Jpn. J. Cancer Res 88:867-876). Referring to the nucleic acid and amino acid

sequence set forth in Figure 10A-10D <sup>(SEQ ID NOS: 11 AND 12)</sup> of this application, one can identify

15 the signal sequence for secretion which is located at the beginning of the sequence and extends to the glycine coded for by nucleotides 76-78. The mature protein begins with Ser-Lys-Leu-Lys, starting at nucleotide 79 of the nucleic acid sequence. Flt1 Ig domain 1 extends from nucleotide 79 to 393, ending with the amino acids Ser-Asp-Thr. Flt1 Ig domain 2 extends

20 from nucleotide 394 to 687 (encoding Gly-Arg-Pro to Asn-Thr-Ile), and Flt1 Ig domain 3 extends from nucleotides 688 to 996 (encoding Ile-Asp-Val to Asp-Lys-Ala). There is a bridging amino acid sequence, Gly-Pro-Gly, encoded by nucleotides 997-1005, followed by the nucleotide sequence encoding human Fc (nucleotides 1006-1701 or amino acids Glu-Pro-Lys to

25 Pro-Gly-Lys-stop).

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A more detailed analysis of the Flt1 amino acid sequence reveals that there is a cluster, namely, amino acid residues 272-281 (KNKRASVRR) of

Figure 10A-10D<sup>(SEQ ID NOS. 11 AND 12)</sup>, in which 6 out of 10 amino acid residues are basic. This

5 sequence is located in Flt1 Ig domain 3 of the receptor (see Figure 11), which is not itself essential for binding of VEGF ligand, but which confers a higher affinity binding to ligand. An alignment of the sequence of Ig domain 3 with that of Ig domain 2 reveals that in this region, there is very poor alignment between the two Ig domains, and that there are about 10

10 additional amino acids in Ig domain 3. An analysis of the hydrophilicity profiles (MacVector computer software) of these two domains clearly indicates the presence of a hydrophilic region in the protein (Figure 12A-

12B). These observations raised the possibility that the actual three dimensional conformation of Flt1 Ig domain 3 allowed for some type of

15 protrusion that is not in Flt1 Ig domain 2. To test this hypothesis, the 10 additional amino acids were deleted and the resulting protein was tested to see whether the deletion would affect the pharmacokinetics favorably without seriously compromising the affinity of the receptor for VEGF.

This DNA construct, which was constructed using standard molecular

20 biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, MA), is referred to as Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc.

25 The Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc construct was derived from Flt1(1-3)-Fc by

(SEQ ID NOS: 11 AND 12)

deletion of nucleotides 814-843 (set forth in Figure 10A-10D), which deletes the highly basic 10-amino acid residue sequence Lys-Asn-Lys-Arg-Ala-Ser-Val-Arg-Arg-Arg from Flt1 Ig domain 3.

- 5 The final DNA construct was sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The sequence of Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc is set forth in Figure 13A-13D. (SEQ ID NOS: 13 AND 14)

10 **Example 12: Construction of Flt1(1-3)-Fc basic region deletion mutant designated Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc.**

A second deletion mutant construct, designated Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc, was derived from the Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc construct by deletion of Flt1 Ig domain 1 encoded by nucleotides 79-393 (see Figure 10A-10D); for convenience, nucleotides 73-78 (TCA GGT) were changed to TCC GGA. (SEQ ID NOS: 11 AND 12)

This introduced a restriction site (BspE1) without altering the associated amino acid sequence, Ser-Gly. This DNA construct, which was constructed using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, MA), was also sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The



sequence of Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc is set forth in Figure 14A-14C.<sup>(SEQ ID NOS: 15 AND 16)</sup>

**Example 13: Construction of Flt1(1-3)-Fc deletion mutant designated Mut3: Flt1(2-3)-Fc.**

5

A third deletion mutate construct, designated Mut3: Flt1(2-3)-Fc, was constructed the same way as the Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc construct, except that Flt1 Ig domain 3 was left intact (the basic region amino acids were not deleted). The construct was constructed using standard molecular

10 biology techniques and the final construct was sequence-verified as described *supra*. The sequence of Mut3: Flt1(2-3)-Fc is set forth in Figure 15A-15C.<sup>(SEQ ID NOS: 17 AND 18)</sup>

**Example 14: Construction of Flt1(1-3)-Fc basic region N-glycosylation mutant designated Mut4: Flt1(1-3<sub>R->N</sub>)-Fc.**

15

A final construct was made in which a N-glycosylation site was introduced into the middle of the basic region of Flt1 Ig domain 3. This construct was designated Mut4: Flt1(1-3<sub>R->N</sub>)-Fc and was made by changing nucleotides

20 824-825 from GA to AC, consequently changing the coded Arg residue (AGA) into an Asn residue (AAC) (see Figure 10A-10D).<sup>(SEQ ID NOS: 11 AND 12)</sup> The resulting

amino acid sequence is therefore changed from Arg-Ala-Ser to Asn-Ala-Ser, which matches the canonical signal (Asn-Xxx-Ser/Thr) for the

addition of a N-glycosylation site at the Asn residue. The sequence of

25 Mut4: Flt1(1-3<sub>R->N</sub>)-Fc is set forth in Figure 16A-16D.<sup>(SEQ ID NOS: 19 AND 20)</sup>

described *infra*. In addition, these molecules were able to bind VEGF tightly as described *infra* and block phosphorylation of the native Flk1 receptor expressed in endothelial cells as described *infra*.

5 **(a) Construction of the expression plasmid**  
**pFlt1D2.Flk1D3.FcΔC1(a)**

Expression plasmids pMT21.Flt1(1-3).Fc (6519bp) and pMT21.Flk-1(1-3).Fc (5230bp) are plasmids that encode ampicillin resistance and Fc-tagged versions of Ig domains 1-3 of human Flt1 and human Flk1, respectively. These plasmids were used to construct a DNA fragment consisting of a fusion of Ig domain 2 of Flt1 with Ig domain 3 of Flk1, using PCR amplification of the respective Ig domains followed by further rounds of PCR to achieve fusion of the two domains into a single fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows:

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3') (SEQ ID NO: 1)

3': Flt1D2-Flk1D3.as (5'-CGGACTCAGAACCACATCTATGATTGTATTGGT-3') (SEQ ID NO: 2)

The 5' amplification primer encodes a BspE1 restriction enzyme site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (corresponding to amino acids 27-33 of Figure 21A-21C). The 3' primer encodes the reverse complement of the 3' end of Flt1 Ig domain 2 fused directly to the 5' beginning of Flk1 Ig domain 3, with the fusion point defined as TIID of Flt1 (corresponding to amino acids 123-126 of

(SEQ ID NOS: 21 AND 22)  
Figure 21A-21C) and continuing into VVLS (corresponding to amino acids  
(SEQ ID NOS: 21 AND 22)  
127-130 of Figure 21A-21C) of Flk1.

For Ig domain 3 of Flk1, the 5' and 3' amplification primers were as  
5 follows:

5': Flt1D2-Flk1D3.s (5'-ACAATCATAGATGTGGTTCTGAGTCCGTCTCATG  
G-3')(SEQ ID NO: 3)

10 3': Flk1D3/apa/srf.as (5'-GATAATGCCCGGGCCCTTTTCATGGACCCTGAC  
AAATG-3')(SEQ ID NO: 4)

The 5' amplification primer encodes the end of Flt1 Ig domain 2 fused  
directly to the beginning of Flk1 Ig domain 3, as described above. The 3'  
15 amplification primer encodes the end of Flk1 Ig domain 3, defined by the  
amino acids VRVHEK (corresponding to amino acids 223-228 of Figure  
(SEQ ID NOS: 21 AND 22)  
21A-21C), followed by a bridging sequence that includes a recognition  
sequence for the restriction enzyme Srf1, and encodes the amino acids  
GPG. The bridging sequence corresponds to amino acids 229-231 of  
(SEQ ID NOS: 21 AND 22)  
20 Figure 21A-21C.

After a round of PCR amplification to produce the individual domains, the  
products were combined in a tube and subjected to a further round of PCR  
with the primers bsp/flt1D2 and Flk1D3/apa/srf.as (described *supra*) to  
25 produce the fusion product. This PCR product was subsequently digested

with the restriction enzymes BspEI and SmaI and the resulting 614bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/ΔB2.Fc, to create the plasmid pMT21/Flt1D2.Flk1D3.Fc.

The nucleotide sequence of the Flt1D2-Flk1D3 gene fusion insert was

5 verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI and the resulting 702bp fragment was transferred into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)B2-FcΔC1(a) to produce the plasmid pFlt1D2.Flk1D3.FcΔC1(a). The complete DNA and deduced amino acid

10 sequences of the Flt1D2.Flk1D3.FcΔC1(a) chimeric molecule is set forth in Figure 21A-21C. (SEQ ID NOS: 21 and 22)

**(b) Construction of the expression plasmid**  
**pFlt1D2VEGFR3D3FcΔC1(a)**

15 The expression plasmid pMT21.Flt1(1-3).Fc (6519bp) encodes ampicillin resistance and an Fc-tagged version of Ig domains 1-3 of human Flt1 receptor. This plasmid was used to produce a DNA fragment containing Ig domain 2 of Flt1 by PCR. RNA from the cell line HEL921.7 was used to produce Ig domain 3 of Flk1, using standard RT-PCR methodology. A

20 further round of PCR amplification was used to achieve fusion of the two Ig domains into a single fused fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows:

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3') (SEQ ID NO: 5)

3': Flt1D2.VEGFR3D3.as(TTCCTGGGCAACAGCTGGATATCTATGATTGTA  
TTGGT) (SEQ ID NO: 6)

The 5' amplification primer encodes a BspE1 restriction site upstream of

5 Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM  
(corresponding to amino acids 27-33 of Figure 22A-22C). The 3'  
amplification primer encodes the reverse complement of the end of Flt1 Ig  
domain 2 fused directly to the beginning of VEGFR3 Ig domain 3, with the  
fusion point defined as TIID of Flt1 (corresponding to amino acids 123-126  
of Figure 22A-22C) and continuing into IQLL of VEGFR3 (corresponding to  
amino acids 127-130 of Figure 22A-22C).

For Ig domain 3 of VEGFR3, the 5' and 3' primers used for RT-PCR were as  
follows:

5': R3D3.s (ATCCAGCTGTTGCCAGGAAGTCGCTGGAGCTGCTGGTA) (SEQ ID NO: 7)  
3': R3D3.as (ATTTTCATGCACAATGACCTCGGTGCTCTCCCGAAATCG) (SEQ ID NO: 8)

Both the 5' and 3' amplification primers match the sequence of VEGFR3.

20 The 296bp amplification product of this RT-PCR reaction was isolated by  
standard techniques and subjected to a second round of PCR to add

suitable sequences to allow for fusion of the Flt1D2 with the Flk1D3 domains and fusion of the Flk1D3 and Fc domains via a GPG bridge (see below). The amplification primers were as follows:

5 5':Flt1D2.VEGFR3D3.s

(TCATAGATATCCAGCTGTTGCCCAGGAAGTCGCTGGAG) (SEQ ID NO: 9)

3': VEGFR3D3/srf.as

(GATAATGCCCGGGCCATTTTCATGCACAATGACCTCGGT) (SEQ ID NO: 10)

10 The 5' amplification primer encodes the 3' end of Flt1 Ig domain 2 fused directly to the beginning (5' end) of VEGFR3 Ig domain 3, as described above. The 3' amplification primer encodes the 3' end of VEGFR3 Ig domain 3, defined by the amino acids VIVHEN (corresponding to amino acids 221-226 of Figure 22A-22C<sup>(SEQ ID NOS: 23 AND 24)</sup>), followed by a bridging sequence that includes a recognition sequence for Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 227-229 of Figure 22A-22C<sup>(SEQ ID NOS: 23 AND 24)</sup>.

20 After one round (for Flt1 Ig domain 2) or two rounds (for Flt4 Ig domain 3) of PCR to produce the individual Ig domains, the PCR products were combined in a tube and subjected to a further round of PCR amplification with the amplification primers bsp/flt1D2 and VEGFR3D3/srf.as described *supra*, to produce the fusion product. This PCR product was subsequently  
25 digested with the restriction enzymes BspEI and SmaI and the resulting



(SEQ ID NOS. 25 AND 26)  
in Figure 24A-24C.

**Example 21: Cell Culture Process Used to Produce Modified Flt1 Receptors**

5

**(a) Cell Culture Process Used to Produce Flt1D2.Flk1D3.FcΔC1(a)**

10 The process for production of Flt1D2.Flk1D3.FcΔC1(a) protein using the  
expression plasmid pFlt1D2.Flk1D3.FcΔC1(a) described *supra* in Example 1  
involves suspension culture of recombinant Chinese hamster ovary (CHO  
K1/E1A) cells which constitutively express the protein product. The cells  
are grown in bioreactors and the protein product is isolated and purified  
by affinity and size exclusion chromatography. The process is provided in  
15 greater detail below.

**Cell Expansion**

20 Two confluent T-225 cm<sup>2</sup> flasks containing the Flt1D2.Flk1D3.FcΔC1(a)  
expressing cell line were expanded by passaging cells into eight T-225 cm<sup>2</sup>  
flasks in medium (GMEM + 10% serum, GIBCO) and incubated at 37°C and  
5% CO<sub>2</sub>. When the flasks approached confluence (approximately 3 to 4  
days) the cells were detached using trypsin. Fresh medium was added to  
protect the cells from further exposure to the trypsin. The cells were  
25 centrifuged and resuspended in fresh medium then transferred to eight  
850 cm<sup>2</sup> roller bottles and incubated at 37°C and 5% CO<sub>2</sub> until confluent.

fragments with N-linked glycosylation sites. The results are summarized in the accompanying Figure 36.<sup>1</sup> (SEQ ID NO: 27)

There are a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to the Fc region. Cys27 has been confirmed to be disulfide bonded to Cys76. Cys121 is confirmed to be disulfide bonded to Cys 182. The first two cysteines in the Fc region (Cys211 and Cys214) form an intermolecular disulfide bond with the same two cysteines in another Fc chain. However, because these two cysteines can not be separated enzymatically from each other, it can not be determined whether disulfide bonding is occurring between same cysteines (Cys211 to Cys211, for example) or between Cys211 and Cys214. Cys216 is confirmed to be disulfide bonded to Cys306. Cys 352 is confirmed to be disulfide bonded to Cys410.

There are five possible N-linked glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a). All five of them are found to be glycosylated to varying degrees. Complete glycosylation was observed at Asn33 (amino acid sequence NIT), Asn193 (amino acid sequence NST), and Asn282 (amino acid sequence NST). In addition, partial glycosylation <sup>is observed</sup> ~~is observed~~ on Asn65 and Asn120. Sites of glycosylation are highlighted by underline in the Figure 36.<sup>1</sup> (SEQ ID NO: 27)





upstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

In still another preferred embodiment, the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

In a preferred embodiment of the invention, the multimerizing component comprises an immunoglobulin domain.

In another embodiment, the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Preferred embodiments include an isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic acid molecule consists of a nucleotide sequence selected from the group consisting of

- (a) the nucleotide sequence set forth in Figure 13A-13D (SEQ ID NOS: 13 and 14);
- (b) the nucleotide sequence set forth in Figure 14A-14C (SEQ ID NOS: 15 and 16);
- (c) the nucleotide sequence set forth in Figure 15A-15C (SEQ ID NOS: 17 and 18);
- (d) the nucleotide sequence set forth in Figure 16A-16D (SEQ ID NOS: 19 and 20);

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(e) the nucleotide sequence set forth in Figure 21A-21C (SEQ ID NOS: 21 and 22);

(f) the nucleotide sequence set forth in Figure 22A-22C (SEQ ID NOS: 23 and 24);

5 (g) the nucleotide sequence set forth in Figure 24A-24C (SEQ ID NOS: 25 and 26); and

(h) a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a), (b), (c ), (d),

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polypeptide which comprises growing cells of the host-vector system under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

- 5 Additional embodiments include a fusion polypeptide encoded by the nucleic acid sequence set forth in Figure 10A-10D (SEQ ID NOS: 11 and 12) or Figure 24A-24C (SEQ ID NOS: 25 and 26) Which has been modified by acetylation or pegylation wherein the acetylation is accomplished with at least about a 100 fold molar excess of
- 10 acetylation reagent or wherein acetylation is accomplished with a molar excess of acetylation reagent ranging from at least about a 10 fold molar excess to about a 100 fold molar excess or wherein the pegylation is 10K or 20K PEG.

- 15 A preferred embodiment includes a method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal the fusion polypeptide described above, including embodiments wherein the mammal is a human, the fusion polypeptide is acetylated or the fusion polypeptide is pegylated.

20

A further embodiments is a fusion polypeptide which specifically binds the VEGF receptor ligand VEGF.

- A preferred embodiment of the invention is a method of blocking blood
- 25 vessel growth in a human comprising administering an effective amount of the fusion polypeptide described above.

- Also preferred is a method of inhibiting VEGF receptor ligand activity in a mammal comprising administering to the mammal an effective
- 30 amount of

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Preferred embodiments include a fusion polypeptide wherein amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor and a fusion polypeptide wherein the amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor.

In yet another embodiment, the fusion polypeptide multimerizing component comprises an immunoglobulin domain including an embodiment wherein the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Preferred embodiments include a fusion polypeptide comprising an amino acid sequence of a modified Flt1 receptor, wherein the amino acid sequence selected from the group consisting of (a) the amino acid sequence set forth in Figure 13A-13D (SEQ ID NOS: 13 and 14); (b) the amino acid sequence set forth in Figure 14A-14C (SEQ ID NOS: 15 and 16); (c) the amino acid sequence set forth in Figure 15A-15C (SEQ ID NOS: 17 and 18); (d) the amino acid sequence set forth in Figure 16A-16D (SEQ ID NOS: 19 and 20); (e) the amino acid sequence set forth in Figure 21A-21C (SEQ ID NOS: 21 and 22); (f) the amino acid sequence set forth in Figure 22A-22C (SEQ ID NOS: 23 AND 24); and (g) the amino acid sequence set forth in Figure 24A-24C (SEQ ID NOS: 25 AND 26).

Another preferred embodiment is a method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal the fusion polypeptide described above.

greater than a 1:1 stoichiometric ratio, both the Flt1(1-3)-Fc and the step-acetylated Flt1(1-3)-Fc proteins are able to saturate the VEGF, regardless of the degree of acetylation.

- 5 **Figure 9.** Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc. Balb/c mice (23-28g) were injected subcutaneously with 4mg/kg of unmodified or 10, 20, 40, 60 and 100 fold excess samples of step-acetylated Flt1(1-3)-Fc (3 mice for unmodified, 10, 20 and 40 fold excess samples and 2 mice for 60 and 100 fold excess samples). The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days and 3 days after injection. The sera were assayed in an ELISA-based assay designed to detect Flt1(1-3)-Fc. The  $T_{max}$  for all of the Flt1(1-3)-Fc proteins tested was at the 6 hour time point but the  $C_{max}$  was as follows: Unmodified Flt1(1-3)-Fc:  $0.06\mu\text{g/ml}$ ; 10 fold excess sample: -  $0.7\mu\text{g/ml}$ , 15 20 fold excess sample -  $2\mu\text{g/ml}$ , 40 fold excess sample -  $4\mu\text{g/ml}$ , 60 fold excess sample -  $2\mu\text{g/ml}$ , 100 fold excess sample -  $1\mu\text{g/ml}$ .

**Figure 10A-10D (SEQ ID NOS: 11 AND 12).** Nucleic acid and deduced amino acid sequence of Flt1(1-3)-Fc.

**Figure 11.** Schematic diagram of the structure of Flt1.

**Figure 12A and 12B.** Hydrophilicity analysis of the amino acid sequences of Ig domain 2 and Ig domain 3 of Flt1.

**Figure 13A-13D (SEQ ID NOS: 13 and 14).** Nucleic acid and deduced amino acid sequence of Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc.

**Figure 14A-14 C (SEQ ID NOS: 15 and 16).** Nucleic acid and deduced amino acid sequence

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Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc.

**Figure 15A-15C (SEQ ID NOS: 17 and 18).** Nucleic acid and deduced amino acid sequence of Mut3: Flt1(2-3)-Fc.

5

**Figure 16A-16D (SEQ ID NOS: 19 and 20).** Nucleic acid and deduced amino acid sequence of Mut4: Flt1(1-3<sub>R->N</sub>)-Fc.

**Figure 17.** Binding of unmodified Flt1(1-3)-Fc, basic region deletion mutant Flt1(1-3)-Fc, and Flt1(1-3)<sub>R->N</sub> mutant proteins in a Biacore-based assay. At the sub-stoichiometric ratio (0.25 μg/ml Flt1(1-3)-Fc of unmodified, acetylated or genetically modified samples vs. 01. μg/ml VEGF), there is insufficient Flt1(1-3)-Fc protein to block binding of VEGF to the Flt1(1-3)-Fc immobilized on the Biacore chip. At 0.5 μg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, the stoichiometric ratio approximates 1:1 and there is an increased ability to block VEGF binding to the Biacore chip. At 1.0 μg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, which is approximately a 10:1 stoichiometric ratio, the Flt1(1-3)-Fc proteins are able to block binding of VEGF to the Biacore chip, but they are not equivalent. Unmodified, acetylated, and Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc are essentially equal in their ability to block VEGF binding, whereas Mut4: Flt1(1-3<sub>R->N</sub>)-Fc is somewhat less efficient at blocking binding.

**Figure 18.** Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc, Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc, and Flt1(2-3) mutant proteins to Matrigel® coated plates. Unmodified Flt1(1-3)-Fc protein binds avidly to these wells, the Mut3: Flt1(2-3)-Fc protein binds somewhat more weakly, the Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc protein binds more weakly still, and the Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc protein shows the best profile, binding more weakly than any of the other mutant proteins. The Mut4: Flt1(1-3<sub>R->N</sub>)-Fc glycosylation mutant protein shows only marginal benefit on the Matrigel assay.

**Figure 19.** Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc, Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc, and Flt1(2-3) mutant proteins in an ELISA-based assay. At the concentrations tested, unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc, Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc, and Flt1(2-3) mutant proteins bind VEGF similarly.

**Figure 20.** Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc, Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc, and Flt1(2-3) mutant proteins. the C<sub>max</sub> for these reagents was as follows: Unmodified Flt1(1-3)-Fc - 0.15μg/ml; 40 fold molar excess acetylated Flt1(1-3)-Fc - 1.5μg/ml; and Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc - 0.7μg/ml.

**Figure 21A-21C (SEQ ID NOS: 21 AND 22).** Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed Flt1D2.Flk1D3.FcΔC1(a).

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**Figure 22A-22C (seq id NOS: 23 and 24).** Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed Flt1D2.VEGFR3D3.FcΔC1(a).

5 **Figure 23.** Extracellular Matrix (ECM) Assay. The results of this assay demonstrate that the Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) proteins are considerably less sticky to the ECM as compared to the Flt1(1-3)-Fc protein.

10 **Figure 24A-24C (SEQ ID NOS: 25 and 26).** Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed VEGFR1R2-FcΔC1(a).

15 **Figure 25A-25C.** Phosphorylation assay. At a 1.5 molar excess of either Flt1(1-3)-Fc , Flt1(1-3)-Fc (A40) or transient Flt1D2Flk1D3.FcΔC1(a) there is complete blockage of receptor stimulation by these three modified Flt1 receptors as compared to control media challenge. In contrast, transient Flt1D2VEGFR3D3.FcΔC1(a) does not show significant blockage at this molar excess, as compared with  
20 VEGF positive control challenge. Similar results are seen in Figure 25B, where the modified Flt receptors are in a 3-fold molar excess to VEGF165 ligand. In Figure 25C, where the modified Flt1 receptors are in a 6-fold molar excess to VEGF165 ligand, transient Flt1D2VEGFR3D3.FcΔC1(a) can now be shown to be partially blocking VEGF165-induced stimulation of cell-  
25 surface receptors.

**Figure 26A-26B.** Phosphorylation assay. Detection by Western blot of



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loaded onto a Superose 12 PC 3.2/30 equilibrated in 6M GuHCl and eluted. Peak #1 represents Flt1D2Flk1D3.FcΔC1(a) and peak #2 represents VEGF165.

5 **Figure 33, Figure 34 and Figure 35.** Size Exclusion Chromatography (SEC) with On-Line Light Scattering. Size exclusion chromatography column with a MiniDawn on-line light scattering detector (Wyatt Technology, Santa Barbara, California) and refractive index (RI) detectors (Shimadzu, Kyoto, Japan) was used to determine  
10 the molecular weight (MW) of the receptor-ligand complex. As shown in Figure 33, the elution profile shows two peaks. Peak #1 represents the receptor-ligand complex and peak #2 represents the unbound VEGF165. MW was calculated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor-  
15 ligand complex. The results of these determinations are as follows: MW of the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex at the peak position is 157 300 (Figure 33), the MW of VEGF165 at the peak position is 44 390 (Figure  
34) and the MW of R1R2 at the peak is 113 300 (Figure 35).

20

**Figure 36 (SEQ ID NO: 27).** Peptide mapping and glycosylation analysis. The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) were determined by a peptide mapping method. There are a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of  
25 them belong to the Fc region. Cys27 is disulfide bonded to Cys76. Cys121 is disulfide bonded to Cys 182. The first two cysteines in the Fc region (Cys211 and Cys214) form

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1998, Bioessays 20(2):156-67). In other cases, the known crystal structure of a protein reveals a cluster of positively charged residues on the surface of a protein, but the residues come from different regions of the primary sequence and are only brought together when the protein

5 folds into its tertiary structure. Thus it is difficult to deduce whether an isolated amino acid residue forms part of a cluster of basic residues on the surface of the protein. However, if there is a cluster of positively charged amino acid residues in the primary sequence, it is not unreasonable to surmise that the residues are spatially close to one

10 another and might therefore be part of an extracellular matrix component binding site. Flt1 receptor has been studied extensively and various domains have been described (see for example Tanaka et al., 1997, Jpn. J. Cancer Res 88:867-876). Referring to the nucleic acid and amino acid sequence set forth in Figure 10A-10D (SEQ ID NOS: 11 and 12) of this

15 application, one can identify the signal sequence for secretion which is located at the beginning of the sequence and extends to the glycine coded for by nucleotides 76-78. The mature protein begins with Ser-Lys-Leu-Lys, starting at nucleotide 79 of the nucleic acid sequence. Flt1 Ig domain 1 extends from nucleotide 79 to 393, ending with the amino acids Ser-

20 Asp-Thr. Flt1 Ig domain 2 extends from nucleotide 394 to 687 (encoding Gly-Arg-Pro to Asn-Thr-Ile), and Flt1 Ig domain 3 extends from nucleotides 688 to 996 (encoding Ile-Asp-Val to Asp-Lys-Ala). There is a bridging amino acid sequence, Gly-Pro-Gly, encoded by nucleotides 997-1005, followed by the nucleotide sequence encoding human Fc (nucleotides 1006-

25 1701 or amino acids Glu-Pro-Lys to Pro-Gly-Lys-stop).

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A more detailed analysis of the Flt1 amino acid sequence reveals that there is a cluster, namely, amino acid residues 272-281 (KNKRASVRR) of Figure 10A-10D (SEQ ID NOS: 11 and 12), in which 6 out of 10 amino acid residues are basic. This sequence is located in Flt1 Ig domain 3 of the receptor (see Figure 11), which is not itself essential for binding of VEGF ligand, but which confers a higher affinity binding to ligand. An alignment of the sequence of Ig domain 3 with that of Ig domain 2 reveals that in this region, there is very poor alignment between the two Ig domains, and that there are about 10 additional amino acids in Ig domain 3. An analysis of the hydrophilicity profiles (MacVector computer software) of these two domains clearly indicates the presence of a hydrophilic region in the protein (Figure 12A-12B). These observations raised the possibility that the actual three dimensional conformation of Flt1 Ig domain 3 allowed for some type of protrusion that is not in Flt1 Ig domain 2. To test this hypothesis, the 10 additional amino acids were deleted and the resulting protein was tested to see whether the deletion would affect the pharmacokinetics favorably without seriously compromising the affinity of the receptor for VEGF. This DNA construct, which was constructed using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, MA), is referred to as Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc. The Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc construct was derived from Flt1(1-3)-Fc by

deletion of nucleotides 814-843 (set forth in Figure 10A-10D (SEQ ID NOS: 11 and 12)), which deletes the highly basic 10-amino acid residue sequence Lys-Asn-Lys-Arg-Ala-Ser-Val-Arg-Arg-Arg from Flt1 Ig domain 3.

5

The final DNA construct was sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The sequence of Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc is set forth in Figure 13A-13D (SEQ ID NOS: 13 and 14).

10

**Example 12: Construction of Flt1(1-3)-Fc basic region deletion mutant designated Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc.**

A second deletion mutant construct, designated Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc, was derived from the Mut1: Flt1(1-3<sub>ΔB</sub>)-Fc construct by deletion of Flt1 Ig domain 1 encoded by nucleotides 79-393 (see Figure 10A-10D (SEQ ID NOS: 11 and 12)); for convenience, nucleotides 73-78 (TCA GGT) were changed to TCC GGA. This introduced a restriction site (BspE1) without altering the associated amino acid sequence, Ser-Gly. This DNA construct, which was constructed using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, MA), was also sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The

sequence of Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc is set forth in Figure 14A-14C (SEQ ID NOS: 15 AND 16).

**Example 13: Construction of Flt1(1-3)-Fc deletion mutant designated Mut3: Flt1(2-3)-Fc.**

A third deletion mutate construct, designated Mut3: Flt1(2-3)-Fc, was constructed the same way as the Mut2: Flt1(2-3<sub>ΔB</sub>)-Fc construct, except that Flt1 Ig domain 3 was left intact (the basic region amino acids were not deleted). The construct was constructed using standard molecular biology techniques and the final construct was sequence-verified as described *supra*. The sequence of Mut3: Flt1(2-3)-Fc is set forth in Figure 15A-15C (SEQ ID NOS: 17 and 18).

**Example 14: Construction of Flt1(1-3)-Fc basic region N-glycosylation mutant designated Mut4: Flt1(1-3<sub>R->N</sub>)-Fc.**

A final construct was made in which a N-glycosylation site was introduced into the middle of the basic region of Flt1 Ig domain 3. This construct was designated Mut4: Flt1(1-3<sub>R->N</sub>)-Fc and was made by changing nucleotides 824-825 from GA to AC, consequently changing the coded Arg residue (AGA) into an Asn residue (AAC) (see Figure 10A-10D). The resulting amino acid sequence is therefore changed from Arg-Ala-Ser to Asn-Ala-Ser, which matches the canonical signal (Asn-Xxx-Ser/Thr) for the addition of a N-glycosylation site at the Asn residue. The sequence of Mut4: Flt1(1-3<sub>R->N</sub>)-Fc is set forth in Figure 16A-16D (SEQ ID NOS: 19 and 20).

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described *infra*. In addition, these molecules were able to bind VEGF tightly as described *infra* and block phosphorylation of the native Flk1 receptor expressed in endothelial cells as described *infra*.

5 **(a) Construction of the expression plasmid**  
**pFlt1D2.Flk1D3.FcΔC1(a)**

Expression plasmids pMT21.Flt1(1-3).Fc (6519bp) and pMT21.Flk-1(1-3).Fc (5230bp) are plasmids that encode ampicillin resistance and Fc-tagged versions of Ig domains 1-3 of human Flt1 and human Flk1,  
10 respectively. These plasmids were used to construct a DNA fragment consisting of a fusion of Ig domain 2 of Flt1 with Ig domain 3 of Flk1, using PCR amplification of the respective Ig domains followed by further rounds of PCR to achieve fusion of the two domains into a single fragment. For Ig  
15 domain 2 of Flt1, the 5' and 3' amplification primers were as follows:

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3')(SEQ ID NO: 1)

20 3': Flt1D2-Flk1D3.as (5'-CGGACTCAGAACACATCTATGATTGTATTGGT-3') (SEQ ID NO: 2). The 5' amplification primer encodes a BspE1 restriction enzyme site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (corresponding to amino acids 27-33 of Figure 21A-21C SEQ ID NOS: 21 and 22)). The 3' primer encodes the reverse  
25 complement of the 3' end of Flt1 Ig domain 2 fused directly to the 5' beginning of Flk1 Ig domain 3, with the fusion point defined as TIID of Flt1 (corresponding to amino acids 123-126 of

Figure 21A-21C) (SEQ ID NOS: 21 and 22) and continuing into VVLS (corresponding to amino acids 127-130 of Figure 21A-21C (SEQ ID NOS: 21 and 22)) of Flk1.

For Ig domain 3 of Flk1, the 5' and 3' amplification primers were as follows:

5': Flt1D2-Flk1D3.s (5'-ACAATCATAGATGTGGTTCTGAGTCCGTCTCATG G-3') (SEQ ID NO: 3)

3': Flk1D3/apa/srf.as (5'-GATAATGCCCGGGCCCTTTTCATGGACCCTGAC AAATG-3') (SEQ ID NO:4)

5

The 5' amplification primer encodes the end of Flt1 Ig domain 2 fused directly to the beginning of Flk1 Ig domain 3, as described above. The 3' amplification primer encodes the end of Flk1 Ig domain 3, defined by the amino acids VRVHEK (corresponding to amino acids 223-228 of Figure 21A-21C (seq ID NOS: 21 AND 22)), followed by a bridging sequence that includes a recognition sequence for the restriction enzyme Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 229-231 of Figure 21A-21C (SEQ ID NOS: 21 and 22).

10

15

After a round of PCR amplification to produce the individual domains, the products were combined in a tube and subjected to a further round of PCR with the primers bsp/flt1D2 and Flk1D3/apa/srf.as (described *supra*) to produce the fusion product. This PCR product was

20

subsequently digested

with the restriction enzymes BspEI and SmaI and the resulting 614bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/ $\Delta$ B2.Fc, to create the plasmid pMT21/Flt1D2.Flk1D3.Fc.

The nucleotide sequence of the Flt1D2-Flk1D3 gene fusion insert was

- 5 verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI and the resulting 702bp fragment was transferred into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)B2-Fc $\Delta$ C1(a) to produce the plasmid pFlt1D2.Flk1D3.Fc $\Delta$ C1(a). The complete DNA and deduced amino acid
- 10 sequences of the Flt1D2.Flk1D3.Fc $\Delta$ C1(a) chimeric molecule is set forth in Figure 21A-21C (SEQ ID NOS: 21 and 22).

**(b) Construction of the expression plasmid**

**pFlt1D2VEGFR3D3Fc $\Delta$ C1(a)**

15

The expression plasmid pMT21.Flt1(1-3).Fc (6519bp) encodes ampicillin resistance and an Fc-tagged version of Ig domains 1-3 of human Flt1 receptor. This plasmid was used to produce a DNA fragment containing Ig domain 2 of Flt1 by PCR. RNA from the cell line HEL921.7 was used to

20 produce Ig domain 3 of Flk1, using standard RT-PCR methodology. A further round of PCR amplification was used to achieve fusion of the two Ig domains into a single fused fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows:

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3')

25 (SEQ ID NO: 5)



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3': Flt1D2.VEGFR3D3.as(TTCCTGGGCAACAGCTGGATATCTATGATTGTA  
TTGGT) (SEQ ID NO: 6)

5 The 5' amplification primer encodes a BspE1 restriction site upstream of  
Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM  
(corresponding to amino acids 27-33 of Figure 22A-22C (SEQ ID NOS: 23  
and 24)). The 3' amplification primer encodes the reverse complement of  
the end of Flt1 Ig domain 2 fused directly to the beginning of VEGFR3 Ig  
domain 3, with the fusion point defined as TIID of Flt1 (corresponding to  
10 amino acids 123-126 of Figure 22A-22C (SEQ ID NOS: 23 and 24)) and  
continuing into IQLL of VEGFR3 (corresponding to amino acids 127-130 of  
Figure 22A-22C (SEQ ID NOS: 23 AND 24)).

15 For Ig domain 3 of VEGFR3, the 5' and 3' primers used for RT-PCR were as  
follows:

5': R3D3.s (ATCCAGCTGTTGCCCAGGAAGTCGCTGGAGCTGCTGGTA) (SEQ ID  
NO: 7)

20 3': R3D3.as (ATTTTCATGCACAATGACCTCGGTGCTCTCCCGAAATCG) (SEQ ID  
NO: 8)

Both the 5' and 3' amplification primers match the sequence of VEGFR3.  
The 296bp amplification product of this RT-PCR reaction was isolated by  
standard techniques and subjected to a second round of PCR to add

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suitable sequences to allow for fusion of the Flt1D2 with the Flk1D3 domains and fusion of the Flk1D3 and Fc domains via a GPG bridge (see below). The amplification primers were as follows:

5 5':Flt1D2.VEGFR3D3.s

(TCATAGATATCCAGCTGTTGCCAGGAAGTCGCTGGAG) (SEQ ID NO: 9)

3': VEGFR3D3/srf.as

(GATAATGCCCGGGCCATTTTCATGCACAATGACCTCGGT) (SEQ ID NO. 10)

10

The 5' amplification primer encodes the 3' end of Flt1 Ig domain 2 fused directly to the beginning (5' end) of VEGFR3 Ig domain 3, as described above. The 3' amplification primer encodes the 3' end of VEGFR3 Ig domain 3, defined by the amino acids VIVHEN (corresponding to amino acids 221-226 of Figure 22A-22C (SEQ ID NOS: 23 and 24)), followed by a bridging sequence that includes a recognition sequence for Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 227-229 of Figure 22A-22C (SEQ ID NOS: 23 and 24).

15

20 After one round (for Flt1 Ig domain 2) or two rounds (for Flt4 Ig domain 3) of PCR to produce the individual Ig domains, the PCR products were combined in a tube and subjected to a further round of PCR amplification with the amplification primers bsp/flt1D2 and VEGFR3D3/srf.as described *supra*, to produce the fusion product. This PCR product was subsequently  
25 digested with the restriction enzymes BspEI and SmaI and the resulting

in Figure 24A-24C (SEQ ID NOS: 25 and 26).

**Example 21: Cell Culture Process Used to Produce Modified Flt1 Receptors**

**(a) Cell Culture Process Used to Produce Flt1D2.Flk1D3.FcΔC1(a)**

The process for production of Flt1D2.Flk1D3.FcΔC1(a) protein using the expression plasmid pFlt1D2.Flk1D3.FcΔC1(a) described *supra* in Example 1 involves suspension culture of recombinant Chinese hamster ovary (CHO K1/E1A) cells which constitutively express the protein product. The cells are grown in bioreactors and the protein product is isolated and purified by affinity and size exclusion chromatography. The process is provided in greater detail below.

**Cell Expansion**

Two confluent T-225 cm<sup>2</sup> flasks containing the Flt1D2.Flk1D3.FcΔC1(a) expressing cell line were expanded by passaging cells into eight T-225 cm<sup>2</sup> flasks in medium (GMEM + 10% serum, GIBCO) and incubated at 37°C and 5% CO<sub>2</sub>. When the flasks approached confluence (approximately 3 to 4 days) the cells were detached using trypsin. Fresh medium was added to protect the cells from further exposure to the trypsin. The cells were centrifuged and resuspended in fresh medium then transferred to eight 850 cm<sup>2</sup> roller bottles and incubated at 37°C and 5% CO<sub>2</sub> until confluent.

fragments with N-linked glycosylation sites. The results are summarized in the accompanying Figure 36 (SEQ ID NO: 27).

There are a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to the Fc region. Cys27 has been confirmed to be disulfide bonded to Cys76. Cys121 is confirmed to be disulfide bonded to Cys 182. The first two cysteines in the Fc region (Cys211 and Cys214) form an intermolecular disulfide bond with the same two cysteines in another Fc chain. However, because these two cysteines can not be separated

enzymatically from each other, it can not be determined whether disulfide bonding is occurring between same cysteines (Cys211 to Cys211, for example) or between Cys211 and Cys214. Cys216 is confirmed to be disulfide bonded to Cys306. Cys 352 is confirmed to be disulfide bonded to Cys410.

There are five possible N-linked glycosylation sites in

Flt1D2.Flk1D3.FcΔC1(a). All five of them are found to be glycosylated to varying degrees. Complete glycosylation was observed at Asn33 (amino acid sequence NIT), Asn193 (amino acid sequence NST), and Asn282

(amino acid sequence NST). In addition, partial glycosylation is observed on Asn65 and Asn120. Sites of glycosylation are highlighted by underline in the Figure 36 (SEQ ID NO: 27).